

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

D2

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/02, 1/37, C07K 14/18 // C12N 15/40		A1	(11) International Publication Number: WO 98/16657 (43) International Publication Date: 23 April 1998 (23.04.98)
<p>(21) International Application Number: PCT/US97/18632</p> <p>(22) International Filing Date: 17 October 1997 (17.10.97)</p> <p>(30) Priority Data: 60/028,817 17 October 1996 (17.10.96) US</p> <p>(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).</p> <p>(72) Inventors: CHIEN, David, Y.; Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608 (US). SELBY, Mark, J.; Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608 (US).</p> <p>(74) Agents: POTTER, Jane, E., R.; Chiron Corporation, Intellectual Property – R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US) et al.</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: PROTEASE REGULATOR SCREENING ASSAY</p> <p>(57) Abstract</p> <p>A polypeptide containing an anchor region, a protease recognition site, and a detectable signal region can be produced recombinantly and directly attached to a solid support. The polypeptide is useful for screening protease regulators, especially protease inhibitors.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		

PROTEASE REGULATOR SCREENING ASSAY

This application claims the priority of U.S. Serial No. 60/028,817 filed October 17, 1996.

FIELD OF THE INVENTION

5 The present invention is directed to polypeptides and methods useful for screening protease regulators such as inhibitors of hepatitis C virus (HCV) proteases.

BACKGROUND OF THE INVENTION

Protease activities have been implicated in many pathological processes of disease conditions, especially in viral infections. Protease regulators, especially protease inhibitors, are strongly desired for their potential therapeutic applications. 10 For example, because HCV NS3 protease cleaves the non-structural HCV proteins which are necessary for HCV replication, the NS3 protease can be a target for the development of therapeutic agents against the HCV virus. Several methods are available in the field for screening protease inhibitors. However, there is a continuing 15 need in the art to develop more effective methods and reagents for protease regulator screening.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for screening a compound capable of regulating protease activity.

It is another object of the invention to provide a polypeptide useful for screening test compounds for protease regulating activity.

It is yet another object of the invention to provide a method of forming a substrate for use in a screening assay for protease regulators.

These and other objects of the invention are provided by one or more of the embodiments provided below.

10 In one embodiment of the invention a method is provided for screening a compound capable of regulating protease activity. According to the method, a protease and a polypeptide are incubated in the presence and absence of a test compound. The polypeptide comprises an anchor region, a protease recognition site, and a detectable signal region. The anchor region is bound to a solid support. The 15 protease recognition site comprises a) a protease cleavable peptide bond, and b) at least 3 amino acids on each side of the cleavable peptide bond. The signal region bound to the solid support is detected. An amount of the signal region bound to the solid support in the presence of the test compound which is greater or less than an amount detected in the absence of the test compound indicates that the test 20 compound is capable of regulating protease activity.

In another embodiment of the invention a polypeptide consisting of a polyamino acid sequence is provided useful for screening test compounds for protease regulating activity. The polypeptide comprises an anchor region, a protease recognition site, and a detectable signal region. The anchor region is capable of

binding to a solid support. The protease recognition site comprises a) a cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavable peptide bond.

In yet another embodiment a method is provided of forming a substrate for use in a screening assay for protease regulators. The method comprises the step of 5 binding a polypeptide to a solid support via a protein-protein interaction. The K_d of the protein-protein interaction is less than 10^{-8} . The polypeptide comprises an anchor region, a protease recognition site, and a detectable signal region. The protease 10 recognition site comprises a) a cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavable peptide bond. The polypeptide binds to the solid support via the anchor region.

The present invention provides the art with an effective and rapid method for screening protease regulators. The invention also provides polypeptides used in the method which can be easily produced recombinantly without any chemical 15 modification. The method can be readily carried out in an automated format and is suitable for large scale drug screens.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic of the assay in the absence of protease cleavage. The polypeptide is anchored on a solid support. Y represents the anchor region, ■ 20 represents the protease recognition site, and ●●●- represents the signal region.

Figure 1B is a schematic of the assay after protease cleavage. The signal region is released from the polypeptide and the solid support.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have discovered a simple method of making substrates useful for screening protease regulators. A substrate is produced in a recombinant microorganism, isolated and purified, and directly attached to a solid support. Thus 5 no *in vitro* chemical reactions are required to produce the assay substrate.

Polypeptides for the assay are solely comprised of amino acids. Thus they can be made recombinantly without subsequent chemical modification. They comprise an anchor region, a protease recognition site, and a detectable signal region (Figure 1). The polypeptide can be made recombinantly using any means known in 10 the art. For example, DNA sequences encoding each region can be ligated together and inserted into an expression vector via routine procedures of molecular biology (Molecular Cloning, A Laboratory Manual, Sambrook, Fritsch, Maniatis, Cold Spring Harbor Laboratory Press, 1989). Any expression vector available in the art, including prokaryotic and eukaryotic vectors, can be used to express the polypeptide 15 of the present invention. One such vector is pEZZ18 (Pharmacia, NJ). An expression vector encoding the polypeptide of the present invention can be used to transform host cells, either prokaryotic or eukaryotic host cells. The transformed host cells can either constitutively express or inducibly to express the polypeptide of the present invention, depending on the promoter used in the expression vector.

20 Methods for purifying and/or isolating polypeptides made in expression systems are readily available in the art. For example, procedures for protein purification from *E. coli* can be found at §17.37 of Molecular Cloning, A Laboratory Manual, second edition, Sambrook, Fritsch, Maniatis, Cold Spring Harbor

Laboratory Press, 1989. Alternatively, the polypeptide can be made via non-recombinant methods, *e.g.*, by chemical synthesis.

The anchor region of the polypeptide allows the polypeptide to be attached to a solid support during screening assays. It includes any peptide fragment that can be bound to a solid support via a protein-protein interaction. The interaction 5 preferably has a K_d of 10^{-8} , 10^{-10} , 10^{-12} , or even 10^{-14} . The K_d of the protein-protein interaction should be less than 10^{-8} .

Several peptide moieties are available in the art which may be used as anchor regions. For example, the anchor region can contain an epitope, an antibody binding 10 region, or a protein capable of binding to another protein. The solid support can be coated with antibodies which bind to the epitope, or other proteins capable of specifically binding the anchor region. Numerous proteins or epitopes are suitable for use in the anchor region, *e.g.*, protein A (Kronvall et al., Immunochemistry, 1970, 7: 124-127; Duhamel et al., J. Immunol. Methods, 1979, 31: 211-217), SOD 15 polypeptide (Porstmann et al., J. Immunol. Methods, 1990, 127: 1-10), and S peptide (Kim et al., Protein Sci., 1993, 2: 348-356).

The anchor region can comprise any length from the minimum region required for protein binding to the entire protein. It can be at the N-terminal or C-terminal portion of the polypeptide, or in between. However, the anchor region must 20 be positioned on the opposite side from the signal region with respect to the protease recognition site. The anchor region should also be positioned to minimize steric hindrance for the protease reaction. Steric hindrance if found, can be reduced by use of linker regions as discussed below.

The protease recognition site comprises a protease cleavable peptide bond flanked on both sides by at least 3 amino acids, preferably amino acids naturally adjacent to the protease cleavable peptide bond. In a more preferred embodiment, the peptide bond is flanked by at least 7 or 8 naturally adjacent amino acids on each side. Sequences of protease recognition sites for a variety of proteases are known in the art, e.g., the sequences of HCV protease cleavage sites NS5A/5B, NS4A/4B, and NS4B/5A. (Grakoui et al., J. Virol., 1993, May, 67(5):2832-2843). One or more copies of a single protease recognition site may be present in the polypeptide. They may be present in a series. In another embodiment, different cleavage sites are present in a series on the polypeptide. In yet another embodiment, polypeptides which contain different cleavage sites are attached to the same solid support.

10 The detectable signal region of the polypeptide is the moiety which is assayed to detect cleavage. The detectable signal region is positioned within the polypeptide on the opposite side of the protease recognition site from the anchor region. Thus, 15 when the protease recognition site is cleaved by a protease, the detectable signal region is released from the solid support.

15 The detectable signal region is a substrate for a detecting reaction. It can comprise an epitope, an antibody binding region, an enzyme, a protein binding domain, or a nucleic acid binding domain. In a preferred embodiment, an epitope 20 is used which comprises the amino acid sequence FLAG (SEQ ID NO:), i.e., the FLAG tag. Other suitable epitopes comprise the amino acid sequence PLRQGGG (SEQ ID NO:). An epitope containing a monotonous series of amino acids such as Glu or His, e.g., the 6-Histidine tag, can also be used in the present invention. The detectable signal region can contain one or more copies of

a signal entity, *e.g.*, epitope, or one or more copies of different signal entities, *e.g.*, different epitopes.

The detectable signal region can be detected directly, *e.g.*, by labeled monoclonal antibodies against epitope tags. In one embodiment, monoclonal antibodies are labeled with Europium and detected directly via time-resolved fluorometry. Time-resolved fluorometry is described in several articles, *e.g.*, Hemmilä, I. *Scand. J. Clin. Lab. Invest.* 48: 389-400, 1988; Hemmilä et al., *Anal. Biochem.* 137:335-43; Lövgren et al., In: *Alternative Immunoassays*, Ed, Collins, W., John Wiley & Sons Ltd, Chichester, UK, pp. 203-217, 1985, all 5 incorporated herein by reference. Alternatively, the primary antibodies can be conjugated to enzymes and detected by assaying for the enzymes.

The detectable signal region can also be detected indirectly, *e.g.*, by labeled secondary antibodies which bind to primary monoclonal antibodies which bind to the epitope tags. Secondary antibodies can be labeled by means of 10 enzymes conjugated to them, *e.g.*, horse radish peroxidase (HRP) or alkaline phosphatase (AP).

A linker region may be used in the polypeptide. A linker region is a segment of the polypeptide located between any other domains, *e.g.*, between the anchor region and the protease recognition site, or between the detectable signal 15 region and the protease recognition site. A linker region comprises additional amino acids which provide flexibility to the polypeptide. These may be desirable to separate the domains of the polypeptide in order to allow access of the protease to the recognition site, or to present the recognition site for more efficient 20

cleavage. Amino acid sequences suitable for use as a linker region are mostly neutral, *e.g.*, Gly-Gly-Gly-Ser (SEQ ID NO: ____). The size of a linker region can vary, depending on the particular polypeptide, from about 2 to about 25 amino acids, preferably from about 2 to about 9 amino acids, most preferably from about 5 to about 8 amino acids.

The solid support of the present invention facilitates high throughput screening. The solid support makes it possible to automate the screening assay without the need for excessive manipulation or transfer of the reaction. The solid support can be of any solid material, preferably plastic, such as polystyrene or polycarbonate. Most preferably it is a multi-compartment vessel for screening multiple candidates at once. The solid support can also be in the form of paramagnetic particles (PMPs), latex particles, paramagnetic latex particles, membranes, polystyrene microbeads, and glass beads.

The polypeptide can be used to screen for protease inhibitors as well as protease activators. The proteases for which a regulator is screened include but are not limited to cellular proteases and viral proteases, *e.g.*, HCV proteases and HIV proteases. Protease inhibitors can be used therapeutically to inhibit viral infection. Protease activators can be useful for enhancing cellular signal transduction associated with proteases. In particular, hepatitis C virus (HCV) has been isolated and characterized. See, *e.g.* EP-318,216B, EP-388232A, and EP-398748. The HCV proteases are also known. See, *e.g.*, US Patents 5,371,017; 5,597,691; and 5,585,258. Test compounds or candidate protease regulators

include libraries of molecules, preferably combinatorial libraries, containing, e.g., small organic chemicals, peptides, or oligonucleotides.

To perform a screening assay, a protease and the polypeptide of the present invention are incubated in the presence and absence of a test compound. The 5 polypeptide can be bound to a solid support either before or after the incubation.

Such incubation is carried out under conditions where the protease can cleave its substrate. HCV NS3 protease requires a co-factor, peptide 4A, in order to cleave the NS4B/5A site in HCV. HCV proteases and their co-factors are described in Steinkühler et al., J. of Virology, pp 6694-6700, 1996; Kim et al., Cell, Vol. 87, 10 pp 343-355, 1996; and WO96/36702, all incorporated herein by reference.

Conditions for other HCV protease cleavages are well characterized in the art. HCV cleavage conditions are exemplified in the examples described below.

Cleavage sites and conditions for other proteases are also known. See, e.g., U.S. Patent 5,434,074.

15 After incubation, the cleaved portion of the polypeptide is washed away and the polypeptide bound to the solid support is subjected to a detecting reaction which determines the amount, presence, or absence of the signal region bound to the solid support. Conversely the amount of signal region which is washed away can be determined. Detection of a greater amount of the signal region bound to 20 the solid support in the presence of the test compound than in the absence of the test compound indicates that the test compound is capable of inhibiting protease activity. Alternatively, detection of a smaller amount of the signal region bound to the solid support in the presence of the test compound than in the absence of the

test compound indicates that the test compound is capable of activating or enhancing protease activity.

The sensitivity of the screening assay is based on several factors including the amount of the protease used and the sensitivity of the detection of the signal region. In order to maximize the sensitivity of the assay for detecting inhibitors, the amount of the protease used should be adjusted to give a very low reading of [REDACTED] the signal region bound to the solid support in the absence of a test compound, *i.e.*, close to 100% cleavage by the protease used. Conversely, for detecting enhancers a very low amount of cleavage is desired in the absence of a test compound.

The following examples are provided for exemplification purposes only and are not intended to limit the scope of the invention which has been described in broad terms above.

Example 1:

The HCV Protease Inhibitor Screening Assay Using NS5A/5B As Cleavage Site Expression Construct And Its Encoded Polypeptide

An expression construct of the present invention was made using standard techniques available in the art. A commercially available vector containing Protein A epitope, *i.e.*, pEZZ18 (Pharmacia, New Jersey) was used as the starting point to build the expression construct.

A fragment encoding HCV NS5 peptide protease target site was inserted in frame into the polylinker region of pEZZ18 so that it is connected at the C-terminal region of Protein A. The NS5 peptide protease target site includes the NS5A and

NS5B cleavage site, *i.e.*, amino acids 2420 and 2421, 7 amino acids at the N-terminal side of the cleavage site, and 8 amino acids at the C-terminal side of the cleavage site.

Another sequence fragment encoding the FLAG tag was inserted in frame at the C-terminal end of the NS5 peptide protease target site. The sequence fragment 5 encodes three FLAG tags alternately spaced with two 4-Glycine spacers.

The resulting expression plasmid construct was expressed in bacteria. Purification of the expression product was carried out via a routine procedure. The overnight bacterial culture was spun down. The supernatant was passed through a 0.45 micron filter and subsequently placed on to an Ig-Sepharose column. The 10 column was eluted with 0.5M Glycine at pH 3.5. The eluted protein was neutralized, concentrated, and desalted using a Centricon-10 spin column.

Coating The Plates

Microwell plates containing 96 wells were prepared by coating the wells with IgG. Then the plates were dried. The purified protein NSS was incubated with the 15 dry plates for 1 hour at 37°C to allow binding of the Protein A region of the protein to the IgG in the microwells.

The Protease

Protease was prepared by elution from a nickel column. Various concentrations of HCV protease were prepared. The protease was incubated with 20 and without a known protease inhibitor, DCIC (di-chloro-iso-coumarin). The incubation was for 1 hour at room temperature. Subsequently the protease was transferred to the coated plates. The incubation was continued overnight at room temperature and then the plate was washed.

The Detection

Diluted monoclonal anti-flag mouse antibodies (available from Kodak) were added to the HCV protease treated plates and incubated with the plates for 1 hour at 37°C. The plates were subsequently washed and incubated with affinity purified goat anti-mouse-HRP (horse radish peroxidase) conjugates for 1 hour at 37°C. Then the plates were washed, and incubated with a developer such as o-phenylenediamine dihydrochloride (OPD). The optical density (OD) was determined at 492 and 620 nm settings.

Experimental Design:

a) Different protease preparations were tested at different concentrations for their protease activity in the absence of a protease inhibitor.

Table 1

50 ng substrate \ well plate	OD of the reaction product			
	1:20 protease dilution	1:50 protease dilution	1:100 protease dilution	protease activity
protease 1	0.036	0.041	0.037	+
protease 2	0.091	0.131	0.201	+
protease 3	0.284	0.265	0.263	±
protease 4 (negative control)	0.263	0.263	0.255	-
protease 5 (negative control)	0.245	0.234	0.242	-
protease 6 (negative control)	0.266	0.280	0.281	-
substrate	0.013	0.009	0.010	-
PBS	0.008	0.005	0.001	-

Table 2

	OD of the reaction product			
200 ng substrate \ well plate	1:20 protease dilution	1:50 protease dilution	1:100 protease dilution	protease activity
protease 1	0.075	0.069	0.091	+
protease 2	0.281	0.412	0.497	+
protease 3	0.770	0.930	0.769	±
protease 4 (negative control)	0.822	0.919	0.780	-
protease 5 (negative control)	0.833	0.870	0.828	-
protease 6 (negative control)	0.847	0.813	0.788	-
substrate	0.011	0.018	0.010	
PBS	0.006	0.006	0.007	

protease 1: Ni-13 (F21) 20 μ M, preparation made at Chiron.

protease 2: HCV P 2/23/96, preparation made at Chiron.

protease 3: NS3 4A (F1), preparation made at Chiron.

protease 4: NS3 4A mnt, (3), mutant protease, preparation made at Chiron.

protease 5: NS3 4A mnt, (2), mutant protease, preparation made at Chiron.

protease 6: CA3, mutant protease, preparation made at Chiron

substrate: pro A-NS5, 1xPBS, polypeptide substrate of HCV protease

The results as shown in tables 1 and 2 demonstrate that protease preparations 1 and 2 are good candidates to be used for the screening assay. For the following experiments, protease preparation 1 was used.

b) Determination of the incubation time and the specificity of the screening assay.

Table 3

Control Setups

	substrate (pro A- NS5)	protease	protease inhibitor (DCIC)	monoclonal anti-flag mouse antibody	goat anti- mouse conjugate
control 1	+	- (PBS)	- (PBS)	- (PBS)	+
control 2	+	- (PBS)	- (PBS)	+	+
control 3	+	+	- (PBS)	+	+
control 4	+	+	+	+	+

Table 4

Incubation time: substrate (proA-NS5) and protease was incubated overnight

	OD (pro A-NS5 10 µg/ml)				OD (proA-NS5 2 µg/ml)			
	pro. 0	pro. 1:100D	pro. 1:200D	pro. 1:500D	pro. 0	pro. 1:100D	pro. 1:200D	pro. 1:500D
control 1	0.042	N/A	N/A	N/A	0.034	N/A	N/A	N/A
control 2	0.913	N/A	N/A	N/A	0.668	N/A	N/A	N/A
control 3	N/A	0.260	0.249	0.364	N/A	0.198	0.182	0.311
control 4	N/A	0.899	0.998	0.887	N/A	0.449	0.467	0.328

Table 5

Incubation time: substrate (proA-NS5) and protease were incubated for 2 hours

	OD (pro A-NS5 10 µg/ml)				OD (proA-NS5 2 µg/ml)			
	pro. 0	pro. 1:100D	pro. 1:200D	pro. 1:500D	pro. 0	pro. 1:100D	pro. 1:200D	pro. 1:500D
control 1	0.004	N/A	N/A	N/A	0.010	N/A	N/A	N/A
control 2	1.209	N/A	N/A	N/A	0.770	N/A	N/A	N/A
control 3	N/A	0.667	0.677	1.488	N/A	0.606	0.352	0.835
control 4	N/A	1.137	1.235	0.503	N/A	0.627	0.631	0.545

Table 6

Incubation time: preincubation of protease, protease substrate, and protease inhibitor overnight

	OD (pro A-NS5 10 µg/ml)			
	protease 0	protease 1:100D	protease 1:200D	protease 1:500D
control 1	0.003	N/A	N/A	N/A
control 2	1.136	N/A	N/A	N/A
control 3	N/A	0.289	0.247	0.315
control 4	N/A	1.578	1.458	1.083

The results as shown in Tables 4-6 demonstrate that the screening assays is specific protease cleavage. The data also demonstrate the dependence of the assay on incubation time of the substrate and the protease.

c) Testing pools 11896 and 11890 of Chiron's compound library for protease inhibiting activity.

Table 7

Protease, protease substrate, and protease inhibitor were preincubated overnight

	Exp.1	Exp.2	[OD(control 4)/OD (test inhibitor)]=I/E	[OD(test inhibitor)-OD(reference inhibitor*)] /OD(test inhibitor)=I%
control 1	0.021	0.066		
control 2	1.234	0.930		
control 3	0.354	0.227		
control 4	1.235	0.768	1.235/0.354=3.5x 0.768/0.227=3.4x	
control 4 with 11896 as the inhibitor (1:100D)	0.471	0.306	1.235/0.471=2.6x 0.768/0.306=2.51x	(0.471-0.354)/0.471=25.7% (0.306-0.227)/0.306=26.2%
control 4 with 11890 as the inhibitor (1:100)	0.387	0.294	1.235/0.387=3.19x 0.768/0.294=2.61x	(0.387-0.354)/0.387=9% (0.294-0.227)/0.294=23.4%

*reference inhibitor: protease inhibitor DCIC which represents 100% inhibition.

Example 2

The HCV Protease Inhibitor Screening Assay Using NS4B/5A Cleavage Site

a) Experimental Procedure

Materials:

Plate: Human IgG coated on HB COSTAR plate, 200 ng/well

Conjugate: Affinity purified goat anti-mouse (H+L)-HRP (Fab)2, (1:5K)

Conjugate Diluent: Ortho conjugate diluent

Protein A-NS5 construct: 10 µg/ml in 1xPBS

Mouse monoclonal anti-FLAG M2 (IBI, IB13010/6j0612): 1:200d in conjugate diluent

Enzyme Stock Buffer: 20mM Tris, 50% glycerol, 150 mM NaCl, 0.2%

Ethyglucoside, (5mM DTT)

The protease: Ni-13 (F21) diluted 1:300 in the enzyme stock buffer

The protease inhibitor: diluted to 1mM in the enzyme stock buffer

5 The pharmaceutical protease inhibitor: diluted to 1 μ M in the enzyme stock buffer

A substrate polypeptide was prepared having an anchor region (protein A), a linker, two protease target sites of NS4B/5A, another linker, and a Flag epitope tag. The target polypeptide was expressed in sufficient quantities to conduct the assay.

10 The substrate polypeptide, (10 μ g/well) in 200 μ l was added to a human IgG coated plate and incubated for one hour. The plate was washed five times with wash buffer. The inhibitor, if any, was pre-incubated (diluted to 1 μ M) with HCV protease, NS4A (0.2 μ M) for one hour at room temperature. Peptide 4A, a co-factor of the NS3 HCV protease was added in different concentrations to all wells containing the protease mixture (20X concentration is 4 μ M). The above mixture was transferred to the plate coated with IgG-4B/5A complex and incubated overnight at room temperature (about 20°C -25°C). The final concentration of the NS4A in each well was 4 μ M. The plate was washed five times with wash buffer. Mouse anti-Flag monoclonal antibody in the amount of 200 μ l (1:200d from stock solution) was added to the designated wells and incubated one hour at 37°C. The plate was washed five times with wash buffer. Affinity purified goat anti-mouse (H+L)-HRP (Fab')₂ conjugate (1:5000d) in the amount of 200 μ l was added to all the reaction wells except the blank well and incubated one hour at 37°C. The plate was washed five times with wash buffer. Then the plate was developed with OPD substrate for

30 minutes at room temperature. The plate was read with a microtiter plate reader at 490/620 nm.

b) Results

Table 8

Substrate 4B/5A: 10 µg/well

	OD of the reaction product		
	no peptide 4A	10X peptide 4A	20X peptide 4A
1X PBS	0.015	0.034	0.016
substrate only	1.645/1.626	1.643/1.570	1.403/1.453
substrate + protease	1.224/1.207	0.706/0.668	0.541/0.539
substrate +protease +DCIC inhibitor (1µM)	1.769/1.686	1.729/1.715	1.584/1.690

Table 9

Substrate 4B/5A: 5 µg/well

	OD of the reaction product		
	no peptide 4A	10X peptide 4A	20X peptide 4A
1X PBS	0.505	0.43	0.039
substrate only	1.357/1.312	1.292/1.187	1.252/1.207
substrate + protease	1.013/0.970	0.564/0.603	0.538/0.543
substrate +protease +DCIC inhibitor (1µM)	1.400/1.491	1.435/1.328	1.374/1.373

These data demonstrates the operability of the screening assay using HCV NS4B/5A protease cleavage site with co-factor peptide 4A.

Example 3**Using NS5A/5B As Substrate To Screen HCV Protease Inhibitor Candidates****a) Experimental Procedure**

A target polypeptide was prepared having an anchor region, a linker region, a protease target site of HCV NS5A/5B, another linker region, and a Flag epitope tag region. The experiment was designed as in Example 2.

b) Results**Table 10**

control setup: since some protease inhibitor candidates were not water soluble and were dissolved in DMSO, controls for DMSO were added.

	pro A-NS5	protease	protease inhibitor (DCIC)	monoclonal anti-flag mouse antibody	goat anti-mouse conjugate	OD w/ DMSO 1µl	ODw/o DMSO
control 1	+	- (PBS)	- (PBS)	- (PBS)	+	N/A	0.010
control 2	+	- (PBS)	- (PBS)	+	+	N/A	2.602
control 3	+	+	- (PBS)	+	+	0.702	0.680
control 4	+	+	+	+	+	2.596	2.915

Table 11

Testing different protease inhibitor pools:

Pharmaceutical Protease Inhibitor Pools (1 μ M)	OD w/DMSO	Mean of OD w/DMSO
Drug 21509	0.765	0.767
	0.769	
Drug 11888	2.141	2.099
	2.057	
Drug 11892	1.786	1.923
	2.060	
Drug 12427	1.042	1.034
	1.027	
Drug 12425	0.865	0.848
	0.831	
Drug 12447	1.558	1.537
	1.516	
Drug 13318	0.780	0.830
	0.880	
Drug 6769	1.037	1.087
	1.138	
Drug 6768	1.080	1.072
	1.064	
Drug 6763	1.209	1.145
	1.082	
Drug 6760	1.136	1.153
	1.171	
Drug 6757	1.421	1.366
	1.311	
Drug 6756	1.313	1.301
	1.290	

Table 12

protease inhibitor pool	library (Chiron)	number of compounds/library pool	inhibition of protease activity %
A	benzodiazepine	500	
1 μ M			23
B	benzodiazepine	500	
1 μ M			35
C	benzodiazepine	500	
1 μ M			19
D	benzodiazepine	500	
1 μ M			20
E	dihydro-isoquinolone I	40	
0.5 μ M			16
F	benzodiazepine	500	
1 μ M			23
G	benzodiazepine	500	
1 μ M			32
H	dihydro-pyridone	27	
1 μ M			64
I	dihydro-isoquinolone II	40	
1 μ M			7
J	beta-lactam V	100	
1 μ M			3
K	Azepinone	40	
1 μ M			44
L	dihydro-isoquinolone II	40	
1 μ M			7
M	dihydro-pyridone	27	
1 μ M			73

Example 4**Using A Short Incubation Time And NS5A/5B As A Substrate To Screen HCV Protease Inhibitors****a) Experimental Procedure**

5

A target polypeptide was prepared having an anchor region (protein A) molecule, a linker, a protease target site of HCV NS5A/5B, another linker, and a Flag epitope tag region. The target polypeptide was expressed in sufficient quantities to conduct the assay.

10 The inhibitor was pre-incubated (diluted to 1 μ M) with HCV protease (0.2 μ M) for 1 hour at room temperature. A solution of NS5A/5B substrate (20 μ g/well) was added to the pre-incubated protease and protease inhibitor mixture, and the total mixture was incubated for 15 minutes. The mixture was transferred to a human IgG coated plate and incubated for 1 hour at 37°C. The plate was washed five times with wash buffer.

15 Two hundred microliter of mouse anti-Flag monoclonal antibody (1:200d from stock solution) was added to the designated wells and incubated for 1 hour at 37°C. The plate was washed five times with wash buffer. Affinity purified goat anti-mouse (H+L)-HRPO (Fab')₂ conjugate (1:5000d) in the amount of 200 μ l was added to all the reaction wells except the blank well and incubate 1 hour at 37°C. The plate 20 was washed five times with wash buffer. The plate was developed with OPD substrate for 30 minutes at room temperature. The plate was read with a microtiter plate reader at 490/620 nm.

b) Results

Table 13

Control setup

	pro A-NS5	pro.	pro. inhibitor (DCIC)	mono. anti-flag mouse antibody	goat anti-mouse conjugate	15 minutes preincubation	1 hour preincubation
control 1	+	- (PBS)	- (PBS)	- (PBS)	+	0.007/0.018	0.000/0.016
control 2	+	- (PBS)	- (PBS)	+	+	1.383/1.557	2.353/2.002
control 3	+	+	- (PBS)	+	+	0.794/0.701	0.580/0.628
control 4	+	+	+	+	+	1.563/1.590	1.991/2.152

Table 14

Testing different protease inhibitor pools with different preincubation time:

pro. inhibitor pools (Chiron)	OD (1 hour* preincubation)	inhibition % (per μ M)	OD (15 minutes* preincubation)	inhibition % (per μ M)
H (300 μ M)	0.640/0.867	20	1.083/1.176	46
E (200 μ M)	0.614/0.598	0	0.912/0.924	20
N (200 μ M)	0.650/0.605	3	0.849/0.950	4
O (200 μ M)	0.570/0.694	4	0.605/0.670	0
P (200 μ M)	1.367/1.258	54	1.491/1.280	77
Q (200 μ M)	1.393/1.230	54	0.788/1.237	26
J (1 mM)	0.642/0.613	4	0.796/0.960	16
R (2 mM)	0.598/0.613	0	0.771/0.869	9
S (2 mM)	0.550/0.667	0	0.686/0.817	0
T (2 mM)	0.685/0.594	5	0.957/0.884	21
U (2 mM)	0.620/0.623	3	0.881/0.982	22
V (200 μ M)	0.645/0.596	3	0.912/1.061	29

*two duplicates are shown

This experiment demonstrates that with more protease inhibition is observed at 15 minutes preincubation than with 1 hour preincubation.

Example 5

Tandem Assay

5 The tandem assay is performed with two substrate polypeptides: (1) a polypeptide having both an HCV NS4B/5A site and an HCV NS4A/4B site and (2) a ~~polypeptide having an HCV NS5A/5B site.~~

10 The first substrate polypeptide includes a protein A anchor region, a linker region, a NS4B/5A target site, a NS4A/4B target site, another linker, and a flag epitope. The second target polypeptide includes a SOD anchor region, a linker region, a NS5A/5B site, another linker region, and a PLRQGGG epitope.

15 The first target polypeptide was incubated with paramagnetic particles (PMP) coated with human IgG at 37°C for 18 minutes to form PMP-1. The second target polypeptide was incubated with PMP coated with anti-SOD monoclonal antibodies at 37°C for 18 minutes to form PMP-2.

20 NS3 HCV protease and 4A peptide in the presence and absence of a protease inhibitor were preincubated for an hour at room temperature and were added into a mixture of PMP-1 bound with first target polypeptide containing 4A/4B and 4B/5A (30 µg/assay) and PMP-2 bound with second target polypeptide containing 5A/5B (30 µg/assay). The total reaction mixture was incubated at room temperature for 18 minutes and then washed 5 times with assay wash buffer. Subsequently, murine monoclonal anti-flag epitope antibody conjugated to 2', 6'-Dimethyl-4'-(N-succinimidylloxycarboxyl)phenyl 10-3'-Sulfopropyl)-acridinium-9-carboxylate (NSP-

DMAE-NHS) (100 μ l of 1:50000 dilution) and murine monoclonal anti-PLRQGGG epitope antibody conjugated to (2', 6'-Dimethyl-4-N-succinimidylloxycarboxnyl)phenyl 5-Methyl-benz[b]acridinium-12-carboxylate Fluorosulfonate (LEAE-NHS), (100 μ l of 1:50000 dilution) were incubated with the 5 reaction mixture for 18 minutes at 37°C. The reaction mixture was then washed five times followed by addition of hydrogen peroxide and NaOH. The relative light units (RLU) were read on Dual Wavelengths Photo Multiplier System made by Ciba Corning Diagnostic, Inc., Walpole, MA. DMAE is detectable at 450 nm wavelength and LEAE is detectable at 600 nm wavelength.

10 **Example 6**

Screening Assay Using Monoclonal Antibodies Directly Labeled With Eu³⁺ and Time-Resolved Fluorometry Detection Technology.

Reagents:

15 Reaction Buffer: 50 mM Hepes (pH 7.6), 3 mM MgCl₂, 50% glycerol (v/v), 1 mM DTT (added fresh), 0.1% BSA (added fresh).

Delfia Wash Buffer: 5 mM Tris (pH 7.6), 0.01% Tween-20, 150 mM NaCl, 1.5 mM NaAzide.

Delfia Assay Buffer: purchased from Wallac, cat# 138-022-91.

Delfia Enhancement Solution: purchased from Wallac, cat# 1244-105.

20 Protease: Ni-Large#18 prep from Mike Chin at Chiron. This construct has a free N-terminus and a C-terminal 6XHIS tag. Stock concentration is 359 μ M. It was diluted 1: 16,320 in the reaction buffer and 180 μ l of which was added into a 200 μ l reaction to reach a final protease concentration of 20 nM.

NS4A peptide: purchased from CPR, cat# CIS 2812, lot#CEO 412. The sequence of the 4A peptide is KKGSVVIVGRIVLSGKPAIIPKK-OH. The stock concentration is 12.5 mM in DMSO and 10 mM DTT. It was diluted 1: 11,360 into diluted protease solution. The final concentration of 4A peptide was 1 μ M.

5 Substrate: protein A-G-G-G-G-7aa-NS4B/5A cleavage site-8aa-FLAG-G-FLAG-G-FLAG. The concentration of the stock solution is 724 μ M. It was diluted 1:3620 in reaction buffer and 20 μ l of which was added into 200 μ l reaction to reach a final concentration of 20 nM.

10 Eu³⁺-anti-FLAG MoAb: purchased from Sigma via Kodak, cat#IB13026, lot# 710413. Eu³⁺ labeling reagent was purchased from Wallac, cat# 1244301. The concentration of the stock solution is 10 μ M. It was diluted 1:1,175 in Delfia assay buffer and 200 μ l of which was added into each reaction well.

15 IgG: purchased from Sigma, cat#I-2511, lot# 027H4804. The concentration of the stock solution is 4.8 mg/ml. It was stored in small aliquots at -70°C. It was thawed once before using in the reaction. The stock solution was diluted 1:320 in PBS and 200 μ l of which was added into each reaction well to reach a final concentration of 15 μ g/ml.

20 Plates: Immulon-4 flat bottom plate was used for IgG coating. It was purchased from Fisher, Dynatech cat# 0110103855. For initial reaction, Falcon 96-well U-bottom plate was used. It was purchased from Fisher.

Plate Sealer: soft plastic seals for 96-well plates, cat#320060, purchased from Fisher.

Procedure

Immulon-4 plates were sealed and incubated with IgG (200 μ l of 15 μ g/ml in PBS) for 2 hours at 37°C. The plates were then washed 5 times with 200 μ l of Delfia wash buffer and blocked by incubating with 200 μ l of low detergent BLOTO for 1 hour at 37°C. Alternatively, the plates were blocked overnight at 4°C.

5 A mixture of protease, 4A peptide, and the reaction buffer was prepared by adding 21.4 μ l of protease stock and 30.8 μ l of 4A peptide stock to 350 ml of reaction buffer. The mixture in the amount of 180 μ l was added into an assay plate. The reaction buffer in the same amount was also added into the assay plate as a negative control. The assay plate was sealed and stayed at room temperature for 1
10 hour.

A substrate solution was prepared by adding 13.8 μ l of the stock into 50 ml of the reaction buffer. The substrate solution in the amount of 20 μ l was added into each well of the assay plate containing the protease and 4A peptide. The plate was once again sealed and kept at room temperature for 1 hour. Subsequently, an aliquot
15 (200 μ l) of the reaction mixture in each well of the assay plate was transferred into the wells of IgG coated Immulon-4 plate. The IgG coated plate containing the reaction mixture of the assay plate was sealed and kept at 37°C for 1 hour. Then it was washed five times with 200 μ l Delfia wash buffer.

Subsequently, a solution of Eu³⁺-anti-FLAG MoAb was prepared by addition
20 of 228 μ l Eu³⁺-anti-FLAG MoAb into 400 ml Delfia assay buffer. The solution was then passed through a 0.45 μ m filter and 200 μ l of which was added into each well of the IgG coated plate. The plate was once again sealed, kept at 37°C for 1 hour, and washed 5 times with 200 μ l Delfia wash buffer. Then the plate with Delfia

enhancement solution (200 μ l) added into each well was gently shaken for 10 minutes and was ready for Eu³⁺ reading.

Results

Table 15

Substrate	Protease	NS4A peptide	DMSO	protease inhibitor (Chiron ID 34602.2)	Eu ³⁺ counts
+			0.50%		1,307,260
+			2.50%		1,256,640
+	+		0.50%		1,103,418
+	+		2.50%		1,111,651
+	+	+	0.50%		291,779
+	+	+	2.50%		369,729
+	+	+		3 μ M	540,263
+	+	+		15 μ M	869,825

The principles, preferred embodiments and modes of operation of the present invention have been described in the foregoing specification. The invention which is intended to be protected herein, however, is not to be construed as limited to the particular forms disclosed, since they are to be regarded as illustrative rather than 5 restrictive. Variations and changes may be made by those skilled in the art without departing from the spirit of the invention.

SYNOPSIS OF THE INVENTION

1. A method for screening a compound capable of regulating protease activity comprising the steps of:
 - 5 incubating in the presence and absence of a test compound, a protease and a polypeptide wherein the polypeptide comprises an anchor region, a protease recognition site, and a detectable signal region, wherein the anchor region is bound to a solid support and the protease recognition site comprises a) a protease cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavable peptide bond,
 - 10 detecting the signal region bound to the solid support, wherein detecting an amount of the signal region bound to the solid support in the presence of the test compound which is greater or smaller than an amount detected in the absence of the test compound indicates that the test compound is capable of regulating protease activity.
- 15 2. The method of item 1 wherein the anchor region specifically binds to an antibody.
3. The method of item 2 wherein the anchor region comprises protein A.
4. The method of item 1 wherein the protease recognition site is a hepatitis C virus (HCV) protease recognition site which is selected from the group consisting of 4A/4B, 4B/5A, 5A/5B, and serial combinations thereof.
- 20 5. The method of item 1 wherein the signal region comprises an epitope.
6. The method of item 5 wherein the epitope comprises the amino acid sequence FLAG (SEQ ID NO ____).

7. The method of item 1 wherein the polypeptide further comprises a linker region between the anchor region and the protease recognition site.

8. The method of item 1 wherein the protease recognition site comprises a) a protease cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavage peptide bond.

9. The method of item 1 wherein the anchor region specifically binds to an antibody, the protease recognition site is a HCV protease recognition site which is selected from the group consisting of 4A/4B, 4B/5A, 5A/5B, and serial combinations thereof, and the signal region comprises an epitope.

10. The method of item 9 wherein the anchor region comprises protein A and the epitope comprises the amino acid sequence FLAG (SEQ ID NO:__).

11. The method of item 10 further comprising a linker region between the anchor region and the protease recognition site.

12. A polypeptide consisting of a polyamino acid sequence, said polypeptide being useful for screening test compounds for protease regulating activity, said polypeptide comprising an anchor region, a protease recognition site, and a detectable signal region, wherein the anchor region is capable of binding to a solid support, the protease recognition site comprises a) a cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavable peptide bond.

13. The polypeptide of item 12, wherein the detectable signal region comprises an epitope.

14. The polypeptide of item 12, wherein the anchor region specifically binds to an antibody.

15. The polypeptide of item 14, wherein the anchor region comprises protein A.
16. The polypeptide of item 12, wherein the protease recognition site is a HCV protease recognition site and is selected from the group consisting of 4A/4B, 5
5 4B/5A, 5A/5B, and serial combinations thereof.
17. The polypeptide of item 12, wherein the detectable signal region comprises the amino acid sequence FLAG (SEQ ID NO: ____).
18. The polypeptide of item 12, wherein the anchor region comprises protein A, the protease recognition site is a HCV protease recognition site selected 10 from the group consisting of 4A/4B, 4B/5A, 5A/5B, and serial combinations thereof, and the epitope comprises the amino acid sequence FLAG (SEQ ID NO: ____).
19. An expression vector comprising a DNA sequence encoding the polypeptide of item 12.
20. The vector of item 19 wherein the expression vector is a prokaryotic 15 expression vector.
21. A host cell transformed with the vector of item 20.
22. The method of item 11 wherein detection of an amount of the signal region on the solid support in the presence of the test compound which is greater than an amount detected in the absence of the test compound indicates that the test 20 compound is capable of inhibiting protease activity.
23. The method of item 11 wherein detection of an amount of the signal region on the solid support in the presence of the test compound which is less than an amount detected in the absence of the test compound indicates that the test compound is capable of enhancing protease activity.

24. A method of forming a substrate for use in a screening assay for protease regulators comprising the steps of:
binding a polypeptide consisting of a polyamino acid sequence to a solid support via a protein-protein interaction, wherein K_d of the protein-protein interaction is no more than 10^{-8} , wherein the polypeptide comprises an anchor region, a protease recognition site, and a detectable signal region, wherein the protease recognition site comprises a) a cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavable peptide bond, and wherein the polypeptide binds to the solid support via the anchor region.

5 10 15 20 25. The method of item 24, wherein the detectable signal region contains an epitope.

26. The polypeptide of item 12 which is bound to a solid support.

27. A method for screening a compound capable of regulating protease activity comprising the steps of:
expressing the polypeptide of item 11, 12, 13, 14, 15, or 16 in a host, isolating the polypeptide, binding the polypeptide to a solid support via a protein-protein interaction, incubating the polypeptide with a protease in the presence and absence of a test compound, detecting the signal region bound to the solid support, wherein detecting an amount of the signal region on the solid support in the presence of the test compound which is greater or smaller than an amount detected in the absence of

the test compound indicates that the test compound is capable of regulating protease activity.

28. The method of item 24 wherein the polypeptide is produced in a host cell.

Claims:

1. A method for screening a compound capable of regulating protease activity comprising the steps of:

incubating in the presence and absence of a test compound, a protease and a polypeptide wherein the polypeptide comprises an anchor region, a protease recognition site, and a detectable signal region, wherein the anchor region is bound to a solid support and the protease recognition site comprises a) a protease cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavable peptide bond,

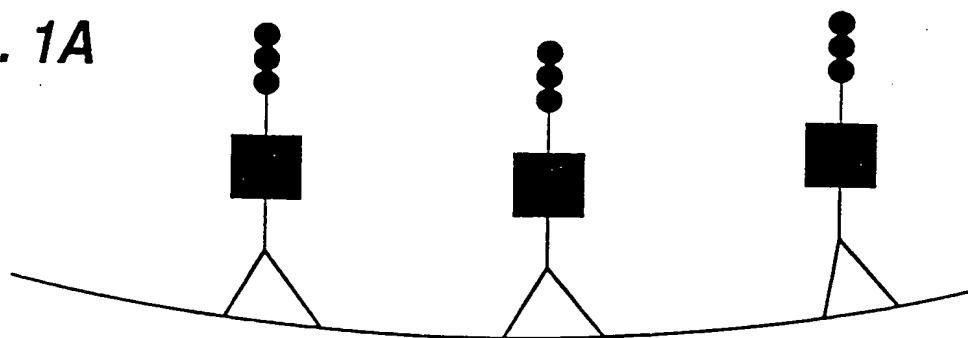
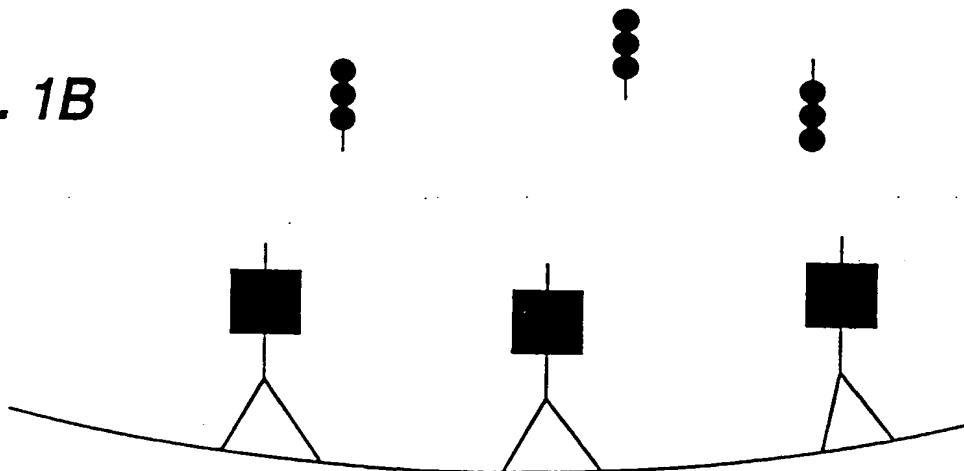
10 detecting the signal region bound to the solid support, wherein detecting an amount of the signal region bound to the solid support in the presence of the test compound which is greater or smaller than an amount detected in the absence of the test compound indicates that the test compound is capable of regulating protease activity.

15 2. A polypeptide consisting of a polyamino acid sequence, said polypeptide being useful for screening test compounds for protease regulating activity, said polypeptide comprising an anchor region, a protease recognition site, and a detectable signal region, wherein the anchor region is capable of binding to a solid support, and the protease recognition site comprises a) a cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavable peptide bond.

20 3. A method of forming a substrate for use in a screening assay for protease regulators comprising the steps of:

binding a polypeptide consisting of a polyamino acid sequence to a solid support via a protein-protein interaction, wherein K_d of the protein-protein interaction is no more than 10^{-8} , wherein the polypeptide comprises an anchor region, a protease recognition site, and a detectable signal region, wherein the protease recognition site comprises a) a cleavable peptide bond, and b) at least 3 naturally adjacent amino acids on each side of the cleavable peptide bond, and wherein the polypeptide binds to the solid support via the anchor region.

1/1

FIG. 1A**FIG. 1B****SUBSTITUTE SHEET (RULE 26)**

INTERNATIONAL SEARCH REPORT

International Application No
PCT 97/18632

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/02 C12Q1/37 C07K14/18 //C12N15/40

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 96 35717 A (SCHERING CORP) 14 November 1996 see page 31 - page 34 ---	1-3
X	US 5 171 662 A (SHARMA SATISH K) 15 December 1992 see page 5 ---	2
X	WO 95 22985 A (ANGELETTI P IST RICERCHE BIO ;FRANCESCO RAFFAELE DE (IT); FAILLA) 31 August 1995 see page 5 ---	2

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

^o Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search

19 March 1998

Date of mailing of the international search report

27/03/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Wells, A

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/18632

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 9642 Derwent Publications Ltd., London, GB; Class B04, AN 96-419825 XP002059091 & JP 08 205 893 A (SUMITOMO METAL IND LTD) , 13 August 1996 see abstract ---	2
X	DATABASE WPI Section Ch, Week 9214 Derwent Publications Ltd., London, GB; Class B04, AN 92-113933 XP002059473 see abstract & US 7 699 466 A (COPELAND T.D. ET AL) 18 February 1992 ---	1,3
A	EP 0 428 000 A (ABBOTT LAB) 22 May 1991 ---	1-3
A	GB 2 276 621 A (MERCK & CO INC) 5 October 1994 see the whole document ----	2
A	WO 95 02065 A (UNIV COLORADO) 19 January 1995 see the whole document -----	1-3

INTERNATIONAL SEARCH REPORT

Internati:	Application No
PCT	97/18632

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9635717 A	14-11-96	AU 5729296 A		29-11-96
		EP 0827508 A		11-03-98
US 5171662 A	15-12-92	NONE		
WO 9522985 A	31-08-95	IT 1272179 B		16-06-97
		AU 1822395 A		11-09-95
		BR 9506931 A		09-09-97
		CA 2182521 A		31-08-95
		EP 0746333 A		11-12-96
		JP 10500005 T		06-01-98
EP 0428000 A	22-05-91	CA 2029123 A		04-05-91
		JP 3172196 A		25-07-91
		JP 6061279 B		17-08-94
GB 2276621 A	05-10-94	US 5436131 A		25-07-95
WO 9502065 A	19-01-95	US 5413914 A		09-05-95
		AU 7254694 A		06-02-95
		US 5691183 A		25-11-97
		US 5627043 A		06-05-97

THIS PAGE BLANK (USPTO)